Cytology of *Sclerotinia sclerotiorum* and Related Species

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Isolates of *Sclerotinia sclerotiorum* (Lib.) de Bary, *S. trifoliorum* Erikss. and *S. minor* Jag. were studied to determine if the numbers of nuclei in mature ascospores and chromosome numbers of these fungi could be used for identification purposes. Isolates of *S. minor* had four nuclei in each ascospore and a haploid chromosome count of four, *S. trifoliorum* had four nuclei in each ascospore and a haploid chromosome count of eight, and *S. sclerotiorum* had two nuclei per ascospore and a haploid chromosome count of eight. Thus, consistent cytological differences were observed, supporting our earlier conclusions that *S. minor*, *S. trifoliorum* and *S. sclerotiorum* are distinct species.

INTRODUCTION

For many years there has been controversy over the taxonomy of the fungi *Sclerotinia sclerotiorum*, *S. trifoliorum* and *S. minor*. After detailed studies of a number of morphological characteristics, Purdy (1955) suggested that these species were conspecific and grouped them into a single species, *S. sclerotiorum*. Recently, we have carried out electrophoretic (Wong & Willetts, 1973, 1975*b*) and mycelial interaction (Wong & Willetts, 1975*a*) studies on these fungi and concluded that *S. minor*, *S. trifoliorum* and *S. sclerotiorum* are distinct species. Cytological observations were not included in our earlier work but it was decided subsequently that cytological data could assist in resolving this taxonomic problem. Björling (1942, 1951) found that the numbers of nuclei in ascospores of *S. sclerotiorum* and *S. trifoliorum* were two and four, respectively, and that the haploid number of chromosomes of both species was six. The latter observation disagreed with the results of Frandsen (1946), who concluded that the haploid chromosome number of *S. trifoliorum* was eight. No cytological studies have been reported for *S. minor*. This paper describes an investigation of the numbers of nuclei in ascospores and chromosome counts on selected isolates of these species. All cultures had been identified previously by means of electrophoretic enzyme patterns.

METHODS

Nineteen isolates of *Sclerotinia* (six of *S. minor*, six of *S. trifoliorum* and seven of *S. sclerotiorum*) were studied. Details of the isolates are given in Table 1.

The isolates were grown on boiled cellophane discs placed on potato dextrose agar medium (Oxoid) in culture plates and incubated at 20 °C under fluorescent light at an intensity of 150 to 200 lux. When vegetative mycelia had grown to appropriate stages for study, pieces of cellophane were cut from the discs, stained and examined under the microscope.

The method described by Purdy (1967) was used for the production of apothecia in the laboratory. Sclerotia of *S. trifoliorum* were incubated at 12 °C and those of the other species were incubated at 15 °C. Apothecia were obtained from most of the isolates, but attempts in the laboratory to produce apothecia from some isolates of *S. trifoliorum* (S6, S7, S33/1 and S76) were unsuccessful. However, ascocarps were obtained from

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Table 1. *Isolates of Sclerotinia spp.*

Species	Isolate no.	Herbarium no.*	Size of sclerotia†	Origin‡
<i>S. minor</i>	S1	—	S	Sunflower, N.S.W.
	S2	ATCC 34323	S	Tomato, N.S.W.
	S3	ATCC 34324	S	Potato, N.S.W.
	S4	—	S	Sunflower, N.S.W.
	S22	—	S	Unknown, N.S.W.
	S77	—	S	Lettuce, Tas.
<i>S. trifoliorum</i>	S6	—	L	Lucerne, N.S.W.
	S7	ATCC 34326	L	Lucerne, N.S.W.
	S33/1	ATCC 34327	L	White Clover, Tas.
	S47	—	L	Red Clover, U.K.
	S48	—	L	White Clover, U.K.
	S76	—	L	White Clover, Tas.
<i>S. sclerotiorum</i>	S8	—	L	French bean, N.S.W.
	S9	DAR 16935	L	French bean, N.S.W.
	S10	—	L	Cauliflower, N.S.W.
	S11	DAR 17481 (= ATCC 34325)	L	Cabbage, N.S.W.
	S12	DAR 26220	L	Sunflower, N.S.W.
	S13	DAR 26223	L	Capsicum, N.S.W.
	S92	—	L	Sclerotium in soil, Tas.

* DAR, Herbarium of the N.S.W. Department of Agriculture, Rydalmere, N.S.W., Australia; ATCC, American Type Culture Collection, Maryland, U.S.A.

† S, Small (0.3 to 2 mm diam.); L, large (1 to 8 mm diam.).

‡ N.S.W., New South Wales, Australia; Tas., Tasmania, Australia.

all these isolates, except S6, when their sclerotia were incubated under field conditions during the Tasmanian autumn.

As ascospores were liberated from apothecia, they were collected on boiled cellophane sheets placed over water agar or nutrient agar media. Ascospores at different stages of development were studied, i.e. ascospores within asci, after discharge and at various stages of germination. Apothecia were cut into small pieces before fixation and staining.

The spores and hyphae on cellophane strips and the apothecial pieces were fixed in ethanol/acetic acid (3:1, v/v). With gentle handling, most of the materials remained on the cellophane throughout the fixing and staining processes. Several nuclear staining techniques were tried, including those which used aceto-carmin, aceto-orcin, Feulgen reagent, iron alum haematoxylin and Giemsa. The Giemsa staining method (C.M.I., 1968) was the most reliable and was used to obtain the results presented below. After staining, the vegetative hyphae and ascospores, still on the cellophane strips, were mounted on microscope slides. Stained pieces of apothecia were teased apart before mounting. Chromosome numbers were counted at anaphase-telophase stages of mitotic division; nuclear numbers were determined in ascospores at the different developmental stages.

RESULTS

General observations

Except for the numbers of chromosomes and the numbers of nuclei in ascospores, the general cytological characteristics of the three *Sclerotinia* species were essentially similar. The vegetative hyphae were multinucleate; in hyphal tips the number of nuclei varied from two to several (Fig. 1*a, b*), while in the older parts of main hyphae more than a hundred nuclei were sometimes observed (Fig. 1*c*). The sizes of nuclei in the older hyphae varied from small fragments consisting of a few chromosomes to some very large nuclei (Fig. 1*d*). The nuclei in young hyphae, hyphal tips, ascospores and germinating ascospores were of a constant size of about 1.5 to 2 μ m (Figs 1*a, b*, 2, 3, 4). Mitotic divisions were frequent and a characteristic feature in all isolates was the synchronous mitotic

Fig. 1. General cytological characteristics of *Sclerotinia* species. (a) Vegetative mycelium of isolate S3 (*S. minor*) showing the multinucleate condition of hyphal cells (n, nucleus). (b) Nuclei at hyphal tips of isolate S8 (*S. sclerotiorum*). (c) Large main vegetative hypha of isolate S8 showing large numbers of nuclei; the cell on the left shows synchronous mitosis of nuclei (m, mitotic figures). (d) Different sizes of nuclei in a large hypha of isolate S8; note the large nuclei (ln). (e) Microconidia of isolate S7 (*S. trifoliorum*); each microconidium contains one nucleus.

Table 2. *Cytological characteristics of Sclerotinia isolates*

<i>Sclerotinia</i> isolate	No. of nuclei in ascospore	Haploid no. of chromosomes
Small-sclerotial isolates		
<i>S. minor</i>		
S3, S22, S77	4	4
S1, S2, S4	No apothecia obtained	4
Large-sclerotial isolates		
<i>S. trifoliorum</i>		
S7, S33/1, S47, S48, S76	4	8
S6	No apothecia obtained	8
<i>S. sclerotiorum</i>		
S8, S9, S10, S11, S12, S13, S92	2	8

division of nuclei in adjacent cells. Most isolates formed microconidia and each microconidium had only one nucleus (Fig. 1e).

Numbers of nuclei in ascospores

Without exception, the number of nuclei was four in undischarged and discharged ascospores of all the isolates of *S. minor* and *S. trifoliorum* that formed apothecia (Figs 2a, 3a; Table 2). This number was present early in the differentiation of ascospores. All isolates of *S. sclerotiorum* had two nuclei in mature ascospores (Fig. 4a; Table 2). Isolates S1, S2, S4 (*S. minor*) and, as stated above, S6 (*S. trifoliorum*) did not produce apothecia.

Chromosome numbers

The haploid number of chromosomes of nuclei in hyphal tips at anaphase–telophase stages of mitosis was four for *S. minor* (Fig. 2b; Table 2) and eight for *S. trifoliorum* and *S. sclerotiorum* (Figs 3b, 4b; Table 2). Counts of chromosomes of dividing nuclei in large hyphae in older parts of colonies were extremely variable and are not given.

Chromosome counts were also made of dividing nuclei in germinating ascospores. These results confirmed the numbers counted in vegetative hyphae.

DISCUSSION

Although the sizes of the nuclei in young hyphae, hyphal tips, ascospores and germinating ascospores were reasonably constant, great variations were observed in older, large hyphae. Nuclei in older hyphae ranged from those with a few chromosomes to those with many. These observations suggest that the nuclei of old vegetative hyphae are unstable, resulting in non-disjunction during mitosis, nuclear fragmentation, non-separation of daughter nuclei and nuclear fusions. Such aneuploidies have been reported in other fungi (Kafer, 1961; Knox-Davies, 1967). Synchronous mitotic divisions frequently occurred in all isolates; Hall (1963) made similar observations with *Monilinia fruticola*.

The cytological data on ascospore nuclear numbers and chromosome numbers is consistent with our earlier separation of these *Sclerotinia* isolates into three species (Wong & Willetts, 1975a, b). The small-sclerotial isolates (*S. minor*) differed from the large-sclerotial isolates (*S. trifoliorum* and *S. sclerotiorum*) in their chromosome numbers (four and eight, respectively), while the last two species differed in the numbers of nuclei in their ascospores (four and two, respectively). Our results confirm those of Björling (1942, 1951) on the numbers of nuclei in ascospores of *S. trifoliorum* and *S. sclerotiorum*, but our haploid chromosome count of eight for both species is contrary to his count of six chromosomes. However, our findings agree with those of Frandsen (1946) who concluded that *S. trifoliorum* has a haploid chromosome number of eight.

Fig. 2

Fig. 3

Fig. 4

Figs 2 to 4. Ascospore nuclear numbers and chromosome numbers of *Sclerotinia* species.

Fig. 2. Small-sclerotial isolate S3 (*S. minor*). (a) Ascospores (s) in ascus (a); each spore has four nuclei (n). (b) Chromosomes at anaphase-telophase stages of mitosis; note the four-chromosome condition of the daughter nuclei (m, mitotic figure; c, chromosome).

Fig. 3. Large-sclerotial isolate S48 (*S. trifoliorum*). (a) Ascospores in ascus, each with four nuclei. (b) Eight-chromosome condition of daughter nuclei at anaphase-telophase stages of mitosis.

Fig. 4. Large-sclerotial isolate S8 (*S. sclerotiorum*). (a) Discharged ascospores, each with two nuclei. (b) Eight-chromosome condition of daughter nuclei at anaphase-telophase stages of mitosis.

The large-sclerotial isolates may have developed from a form similar to the present-day *S. minor* by a doubling of chromosome numbers. Such polyploidization would result in changes in form and physiology, of which an increase in sclerotial size could be one of the most obvious. As we found in our previous studies (Wong & Willetts, 1975*a, b*), *S. trifoliorum* appears to be in an intermediate position between *S. minor* and *S. sclerotiorum*.

It is concluded that the numbers of nuclei in ascospores and haploid chromosome numbers can be used to distinguish between *S. minor*, *S. trifoliorum* and *S. sclerotiorum*. These results add further support to the separation of these fungi into three distinct species.

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